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Genetic diversity of *Mycobacterium tuberculosis* isolates obtained from patients with pulmonary tuberculosis in Beira city, Mozambique

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ABSTRACT

Background: Tuberculosis (TB) represents a serious public health problem in Mozambique, with an estimated incidence rate of 548 cases per 100,000 population in 2011. Information on the molecular epidemiology of *Mycobacterium tuberculosis* (MTB) strains circulating in Mozambique is limited. This study provides the first description of the genetic diversity of MTB strains circulating in Beira city, the second largest town in Mozambique.

Methods: A total of 67 MTB isolates were tested to determine genetic lineages and diversity. The genetic lineages were determined using real-time PCR while genetic diversity was assessed by obtaining Mycobacterial Interspersed Repetitive Unit-Variable Numbers of Tandem Repeat profiles.

Results: Only three of the six major lineages were represented, with 41 (61%) strains belonging to lineage 1, 25 (37%) belonging to lineage 4 and the remaining isolate belonging to lineage 3. No lineage 2 strains (containing the Beijing family) were identified. A high degree of diversity amongst the strains from both lineages 1 and 4 were observed. Comparison of the profiles of representative strains with those of reference strains in the MIRU-VNTRplus database revealed that all lineage 1 isolates clustered with the Eastern African Indian (EAI) 5 sub-family. The lineage 4 strains clustered with a variety of different sub-family strains, including the Latin-American-Mediterranean (LAM) 1 sub-family, the Haarlem, Uganda 1 and Cameroon sub-families and the T2-S sub-family.

Conclusions: The TB epidemic in Beira city is caused by a diverse group of MTB strains predominantly belonging to lineages 1 and 4.

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Introduction

Tuberculosis (TB) represents a serious public health problem, and it is estimated that in 2011 more than 8 million cases occurred worldwide [1]. Mozambique, with an estimated incidence rate of 548 cases per 100,000 population in 2011, is one of the countries with the highest TB burden [1]. Mozambique also faces a severe human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) epidemic and in 2009 the HIV prevalence in the adult population was estimated at 11.5% [2]. Among TB patients, the prevalence of HIV is estimated at 64% [3].

The advent of molecular techniques for genotyping has greatly contributed to a better understanding of the epidemiology, evolution and phylogeny of *Mycobacterium tuberculosis* (MTB) [4]. Several molecular typing techniques have been developed, including the *IS6110*-based restriction fragment length polymorphism (RFLP) typing method [5], spoligotyping and Mycobacterial Interspersed Repetitive Units-Variable-Number of Tandem Repeats (MIRU-VNTR) [6]. MIRU-VNTR is based on polymerase chain reaction (PCR) amplification of specific regions of the MTB genome. It identifies specific genotypic profiles by comparing the number of repeats of short deoxyribonucleic acid (DNA) sequences within different loci [6].

Despite the huge TB burden in Mozambique, there is only one published study in the international literature that describes the genetic diversity of MTB in the country [7]. However, that study did not include isolates from any of the four Central region provinces. In this study the genetic lineages of 67 MTB isolates from TB patients, domiciled in Beira city, were determined based on the presence of lineage-defining genomic deletions [6]. Subsequently, MIRU-VNTR profiles were determined to examine the diversity of the MTB strains circulating among this patient population.

Materials and methods

Setting

This study was carried out in Beira city, Mozambique during the month of November, 2009. Beira city is the second largest town in Mozambique and is the capital of Sofala province, located in the Central region of the country. The city has a population of 431,965 inhabitants. TB treatment is only available through the public sector, and it is offered in six health facilities, referred to as TB clinics.

Patients

The patients in this study were sourced from four TB clinics, which accounted for more than 75% of the TB notifications in Beira city. Patients were consecutively recruited for this study if they had pulmonary TB (both smear-positive and smear-negative), if they were at least 18 years old at the time of enrolment, and if they were residents of Beira city. The study included both patients being treated for the first time (new patients) and those with a history of previous TB treatment (re-treatment patients).

Laboratory procedures

All recruited patients were required to provide two sputum samples. After routine microscopy using the Ziehl-Neelsen method, sputum samples were refrigerated at 2–8 °C until they were shipped to the TB National Reference Laboratory (NRL) in Maputo city (capital of Mozambique), within three days of collection.

At the NRL, culture on Lowenstein–Jensen media was performed following World Health Organization (WHO) guidelines [8]. All positive cultures were sent to the Victorian Infectious Diseases Reference Laboratory in Melbourne, Australia for molecular testing. DNA was extracted from the original culture isolates using the FastDNA® SPIN Kit and the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA). The original cultures were also sub-cultured for purification and storage purposes. The DNA was used to determine the genetic lineage by performing PCR and real-time PCR assays to detect the presence or absence of informative regions of difference (RD9, TBD1, RD239, RD750, *pks*15/1 7 bp deletion), using primer pairs previously described [6]. The basis for lineage assignment is shown in Table 1.

The extracted DNA from the MTB isolates was also used for Mycobacterial Interspersed Repetitive Unit-Variable Numbers of Tandem Repeat (MIRU-VNTR) typing using the most discriminatory loci for the lineages identified, according to an approach proposed by Comas et al. [6]. The loci amplified were Mtub04, QUB-11B, Mtub21, QUB-26, Mtub39, ETRA and MIRU40 for the pink lineage strains while the loci Mtub04, QUB-11B, Mtub21, QUB-26, Mtub30, Mtub39, ETRA, MIRU10 and MIRU40 were amplified to type the red lineage [6]. The amplified fragments were analyzed by electrophoresis through 2% agarose gels, with the number of repeats estimated on the basis of the fragment sizes. Analysis of the MIRU-VNTR profiles using the Bionumerics® software, version 6.5 (2010 Applied Maths, NV, Belgium), enabled a comparison of profiles via the generation of Unweighted Pair group Method using Arithmetic averages (UPGMA) trees and identification of clusters of strains of MTB. A cluster was defined as two or more strains of the same lineage with identical MIRU-VNTR profiles.

In order to compare these strains with those described elsewhere, a sub-sample of 22 strains with representative profiles based on the most discriminatory loci were further typed using the 12 MIRU-VNTR loci described by Supply et al. [9] (MIRU2, MIRU4, MIRU40, MIRU10, MIRU16, MIRU20, MIRU23, MIRU24, MIRU26, MIRU27, MIRU31 and MIRU39). The profiles obtained were compared with those in the MIRU-VNTRplus database (http://www.miru-vntrplus.org/) by performing an Identification by Similarity Search, generating Neighbor-Joining trees [10].

Statistical methods

Laboratory data was double entered using Microsoft Excel and then exported to *Stata Intercooled*, version 10 (Stata Corporation, College Station, Texas) where it was merged with demographic and clinical data. Descriptive statistics such as mean, median and standard deviation were used to summarize numerical variables, and frequency tables were used to summarize discrete variables.

Table 1 – Defining genetic characteristics of the six main *M. tuberculosis* complex lineages.

Lineages	Regions					
	TbD1	RD9	RD105	RD750	RD239	*pks* 7 bp
Lineage 1 (pink)	+	+	+	+	–	+
Lineage 2 (blue)	–	+	–	+	+	+
Lineage 3 (purple)	–	+	+	–	+	+
Lineage 4 (red)	–	+	+	+	+	–
Lineages 5 and 6 (brown, yellow, green)	+	–	+	+	+	+

+ Indicates that the region is present.
– Indicates that region is deleted.

This study was part of a larger research project aimed at describing the prevalence of multidrug resistant MTB strains among patients with pulmonary TB in Beira city. The Mozambican Bio-Ethical Committee approved the research project (reference number: 149/CNBS/2009) and the Ministry of Health granted administrative approval. Only participants that provided written informed consent were included in the study.

Results

Patients and isolates

During the study period, 116 patients met the inclusion criteria for this study and sputum samples were obtained from 90 of them. These samples were submitted for mycobacterial culture on Lowenstein-Jensen solid media. MTB was isolated from 67 samples, and the remaining 23 were culture negative. MTB DNA was extracted from the 67 positive cultures.

Among the 67 MTB culture positive patients, 43 (64.2%) were males and 24 (35.8%) were females. The median age was 32 years (range: 18–62 years). HIV status was available for all patients and 46 (74.5%) of them were HIV positive; 35 patients (52.2%) had newly diagnosed smear-positive disease, 19 (28.4%) had newly diagnosed smear-negative disease, and 13 (19.4%) were re-treatment patients.

Determination of MTB strain lineages

Major lineage determination of the 67 isolates was based on the presence or absence of genomic deletions as described by Comas et al. [6] and summarized in Table 1. On this basis, 41 were classified as lineage 1 (pink), corresponding to the East-African-Indian (EAI) family; 25 isolates belonged to lineage 4 (red), which contains several sub-lineages including the Latin-American-Mediterranean (LAM), Haarlem, X and Uganda families; the remaining isolate was classified as lineage 3 (purple), corresponding to the Central Asian spoligotype (CAS) family.

Determination of MTB strain diversity

The MIRU-VNTR profiles, using the most discriminatory loci for the pink and red lineages [6], were determined for 64 strains (for which sufficient DNA was available). For each lineage, the profiles were compared by generating trees using the UPGMA algorithm (see Figs. 1 and 2). Amongst the 39 pink lineage strains analyzed, 26 different profiles were identified with 20 isolates included in seven clusters (Fig. 1). Within the red lineage (25 strains), 15 different profiles were identified and 15 isolates were included in 5 clusters (Fig. 2). One pink lineage strain showed consistently (for all loci) evidence of two bands, suggesting the presence of a mixed infection. This isolate was from a 47-year-old HIV-positive female patient with smear-negative disease.

In order to compare the profiles of the Mozambican strains with profiles present in the MIRU-VNTRplus database, further typing was performed on 22 isolates, 13 from the pink lineage and 9 from the red lineage, representing the diversity of profiles identified. The 12 MIRU-VNTR locus profiles obtained were then compared with those of reference strains present in the MIRU-VNTRplus database. As shown in Fig. 3, the pink lineage strains formed a cluster with the most closely related reference strain (6538/03) belonging to the EAI5 sub-family. In contrast, the red lineage strains showed greater diversity with six strains clustering with the LAM1 sub-family (Fig. 4A). One red lineage strain was related to the T2-S sub-family (Fig. 4A) and two were related to the Haarlem, Uganda1 and Cameroon sub-families (Fig. 4B).

Discussion

This study describes the genetic diversity of MTB strains circulating among patients with pulmonary TB in Beira city. It represents the first report describing the genetic lineages of MTB strains from Central Mozambique.

The identification and characterization of genotype families using real-time PCR and MIRU-VNTR showed that the MTB strains circulating in Beira city are a mix of lineage 1 (pink) and lineage 4 (red) strains, with a predominance of the EAI5 and LAM1 subfamilies. A minority of red lineage strains were related to other sub-families, such as the T2-S and Haarlem. The predominance of EAI5 and LAM1 strains in Beira city is consistent with the results of a recent study carried out by Viegas et al. in seven Northern and Southern provinces of Mozambique. This study identified several spoligotype families with the predominance of the LAM and EAI families which were found in all seven provinces [7].

The LAM and EAI families are also the predominant families in all countries that share a border with Mozambique, for whom genotype data is available. For instance, a study carried out in Tanzania found that the CAS family was the most

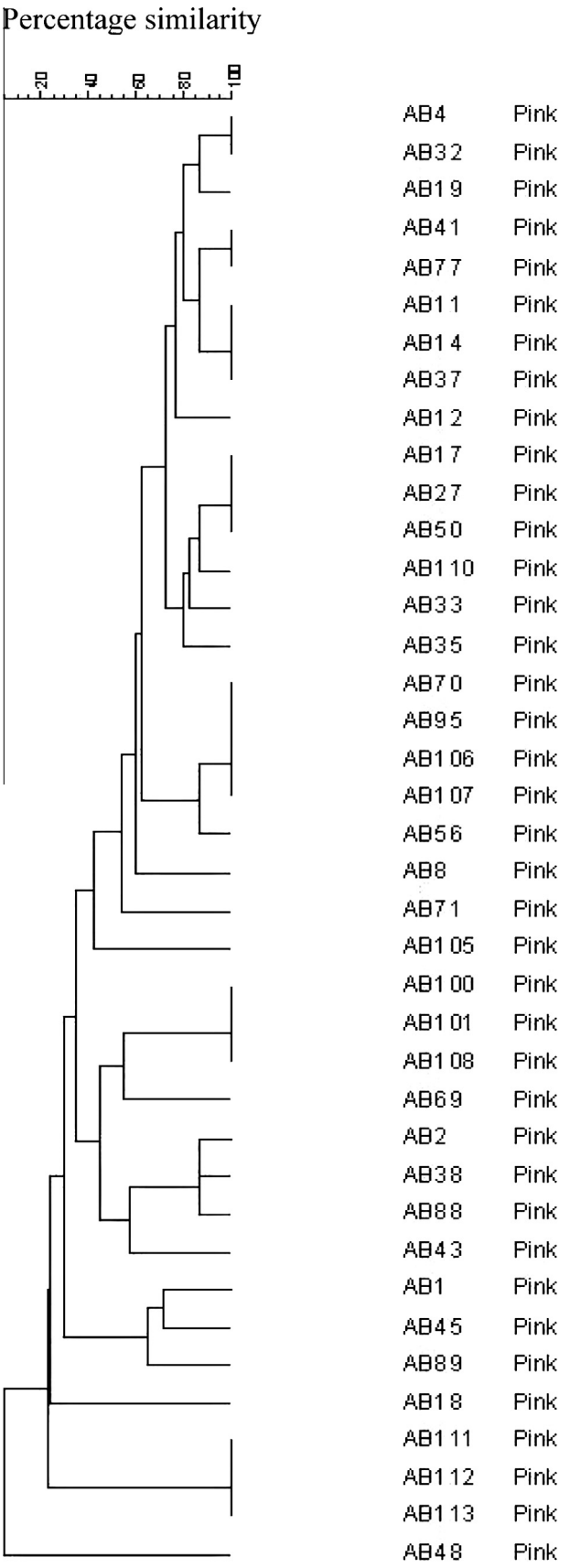


Fig. 1 – UPGMA tree showing the relatedness of the pink lineage strains, based on MIRU-VNTR profiles. The vertical lines link the strains that share the same profile. The first column to the right of the dendrogram shows the laboratory codes used to name the isolates.

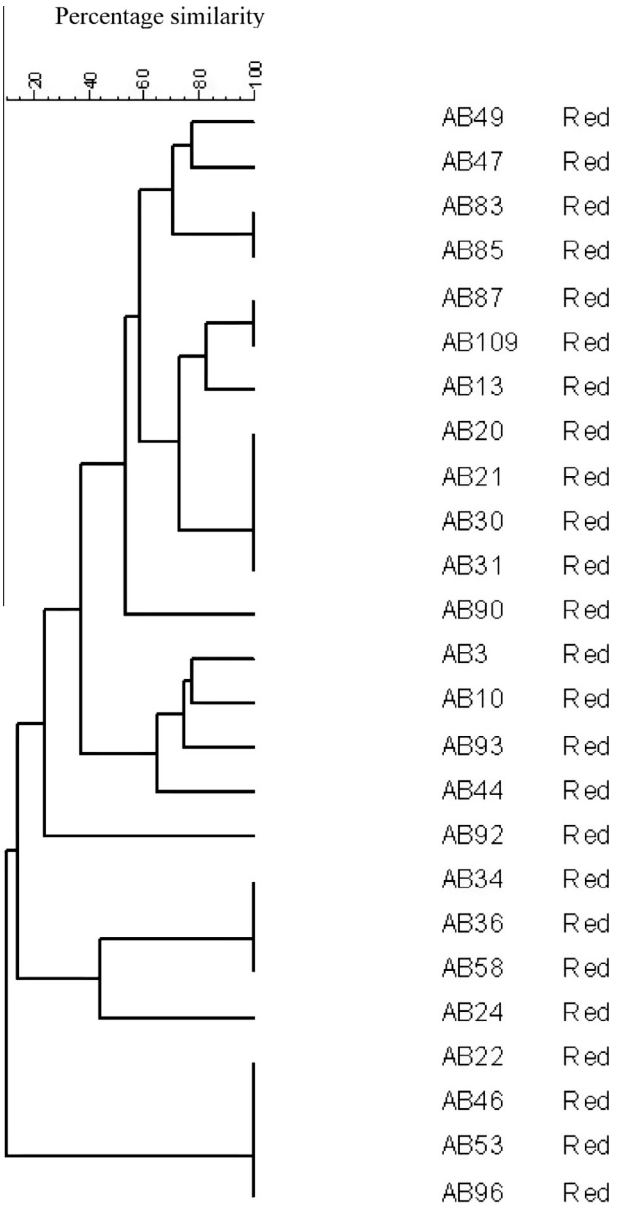


Fig. 2 – UPGMA tree showing the relatedness of the red lineage strains, based on the MIRU-VNTR profiles. The vertical lines link the strains that share the same profile. The first column to the right of the dendrogram shows the laboratory codes used to identify the isolates.

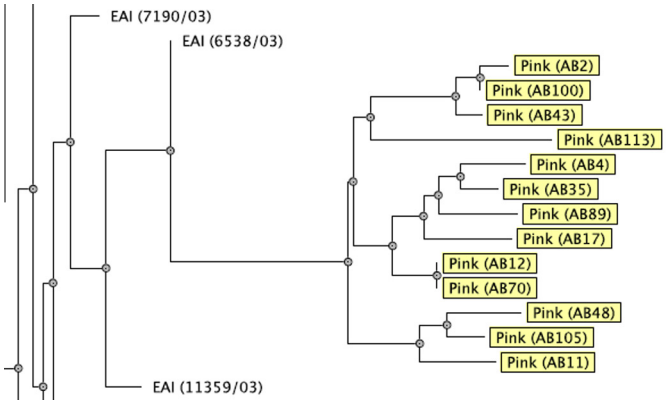
predominant family followed by the LAM and EAI families [11]. Other studies carried out in Zimbabwe and Zambia found a predominant group of strains infecting a considerable proportion of patients with a special spoligotype signature. They called these strains the Southern African 1 (SAF1) family, which had been previously shown to be a member of the LAM family [12,13]. In Malawi, a large study that analyzed the changes in MTB genotypes over a 20-year period found that three quarters of the strains belonged to Lineage 4, and most of them were from the LAM family; the EAI family accounted for almost 8% of the strains [14]. In a national study carried out in South Africa, the LAM family was the most prevalent family in four of the eight provinces surveyed [15]. However, a study carried out in Ethiopia found that more than three quarters of the isolates belonged to three dominant families, namely the T, Manu, and CAS families. The EAI and LAM families played a minor role in this setting [16]. These findings highlight the fact that the distribution of MTB strains varies from region to region.

In contrast with these findings, Viegas et al. found strains of the Beijing family in all four Southern provinces and in two of the three Northern provinces of Mozambique [7]. This difference raises interesting questions about the relative geographical distribution of genotype families in Mozambique. The absence of Beijing strains in this study is consistent with data from neighboring Zimbabwe and Zambia, where no strains of the Beijing family were found in recent studies [12,13]. Conversely, a high predominance of the Beijing family was described in South Africa [15,17], which shares a border with the Southern region of Mozambique, where more Beijing strains were found by Viegas et al. [7]. Strains of the Beijing family have also been described in the Karonga district in Northern Malawi where until 2003 they accounted for less than 8% of all the strains found in that district [18]. A low prevalence of the Beijing family was also observed in Tanzania [11]. Malawi and Tanzania share borders with Northern Mozambique, where low levels of prevalence of the Beijing strain were found by Viegas et al. [7]. Migration plays an important role in the spread of TB [19], and it is possible that the differences in the geographical distribution of genotype strains observed in Mozambique are a reflection of different migration patterns across the country. It is noteworthy that the main transport routes run to the sea across Mozambique rather than North–South within Mozambique, and that it was only recently (in the 1950s) that a North–South road was ever built. It is only since independence (actually, since the civil war ended, in 1992) that much North–South travel actually occurs within Mozambique.

Whilst the pink lineage strains were likely to belong to the EAI5 subfamily, the diversity of MIRU-VNTR profiles suggests that these strains are likely to have evolved from a single strain, which has been in circulation in Beira city for a long period. This is also likely to be the case for the LAM1 family strains; however, the presence of additional unrelated red lineage strains indicates that TB has been introduced into Beira city from several different sources.

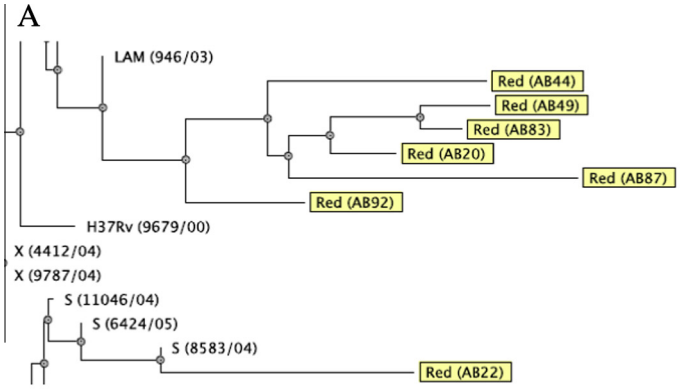
In this study, only one strain of the purple lineage was found. Purple lineage strains are related to the CAS family and the low proportion of these strains observed in this study is consistent with the findings of the Viegas et al. study, in which this family accounted for 2% of all strains [7]. The absence of *Mycobacterium africanum* or *Mycobacterium bovis* strains in this study is also consistent with the findings by Viegas et al. [7], suggesting that these strains do not play a major role in the TB epidemic in Mozambique.

The most important limitation of this study was its small sample size, which may have limited the ability to identify all circulating strains in Beira city. In addition, this study was carried out in primary healthcare clinics and involved patients with less severe disease, so if a potential association between disease severity and genotype family existed, it may have been missed by this study. Finally, in this study, detailed patient data was not collected that could have been used to determine

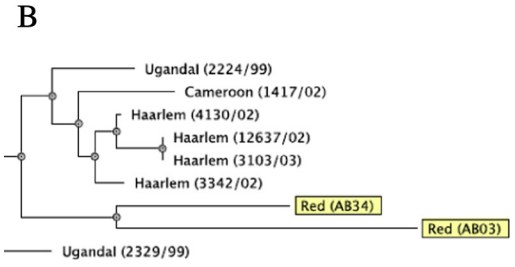


7190/03	EAI	M. tuberculosis	110–41	591	EAI6_BGD1
6538/03	EAI	M. tuberculosis	118–48	1628	EAI5
AB2	Pink	M. tuberculosis	?–?		
AB100	Pink	M. tuberculosis	?–?		
AB43	Pink	M. tuberculosis	?–?		
AB113	Pink	M. tuberculosis	?–?		
AB4	Pink	M. tuberculosis	?–?		
AB35	Pink	M. tuberculosis	?–?		
AB89	Pink	M. tuberculosis	?–?		
AB17	Pink	M. tuberculosis	?–?		
AB12	Pink	M. tuberculosis	?–?		
AB70	Pink	M. tuberculosis	?–?		
AB48	Pink	M. tuberculosis	?–?		
AB105	Pink	M. tuberculosis	?–?		
AB11	Pink	M. tuberculosis	?–?		
11359/03	EAI	M. tuberculosis	120–50		

Fig. 3 – Neighbor-Joining tree comparing the pink lineage strains with reference strains of the MIRUVNTRplus database. The study strains are highlighted in yellow.



946/03	LAM	M. tuberculosis	127–56	20	LAM1
AB44	Red	M. tuberculosis	?–?		
AB49	Red	M. tuberculosis	?–?		
AB83	Red	M. tuberculosis	?–?		
AB20	Red	M. tuberculosis	?–?		
AB87	Red	M. tuberculosis	?–?		
AB92	Red	M. tuberculosis	?–?		
9679/00	H37Rv	M. tuberculosis	153–64	451	H37Rv
4412/04	X	M. tuberculosis	142–15	119	X1
9787/04	X	M. tuberculosis	142–15	119	X1
11046/04	S	M. tuberculosis	131–58	34	S
6424/05	S	M. tuberculosis	139–60	34	S
8583/04	S	M. tuberculosis	141–61	784	T2–S
AB22	Red	M. tuberculosis	?–?		



2224/99	Ugandal	M. tuberculosis	34–16	125	T2
1417/02	Cameroon	M. tuberculosis	75–26		
4130/02	Haarlem	M. tuberculosis	83–15	487	H3
12637/02	Haarlem	M. tuberculosis	90–31	2	H2
3103/03	Haarlem	M. tuberculosis	91–31	2	H2
3342/02	Haarlem	M. tuberculosis	86–29		
AB34	Red	M. tuberculosis	?–?		
AB03	Red	M. tuberculosis	?–?		
2329/99	Ugandal	M. tuberculosis	35–15		

Fig. 4 – (A and B) Neighbor-Joining trees comparing the red lineage strains with reference strains of the MIRUVNTRplus database. The study strains are highlighted in yellow.

whether the clustered cases were epidemiologically linked. Despite these limitations, this study provides useful information about the MTB families circulating in Beira city and complements the findings of Viegas et al., allowing for a full picture of the families circulating in Mozambique to appear.

Conclusions

The results of this study showed that the TB epidemic in Beira city is caused by a mix of lineage 1 (pink) and lineage 4 (red) strains. The pink lineage strains were closely related to the EAI5 sub-family, while most of the red lineage strains were closely related to the LAM1 sub-family. The Beijing family does not seem to play a major role in this setting. The genetic diversity identified within the pink (ancestral) strains indicates that these strains have been in circulation in Beira city for a long period, whereas the red (modern) strains may have been introduced from several sources at different times.

Conflict of interest

We have no conflict of interest to declare.

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